



**UNIVERSIDADE ESTADUAL DE CAMPINAS  
FACULDADE DE ODONTOLOGIA DE PIRACICABA**

**ANDRÉIA BORGES SCRIBONI**

**CARACTERIZAÇÃO E EFEITO DA ENCAPSULAÇÃO DA VANCOMICINA  
EM LIPOSSOMAS SOBRE O BIOFILME DE *Staphylococcus aureus***

**CHARACTERIZATION AND ANTIBIOFILM ACTIVITY OF LIPOSOME-  
ENCAPSULATED VANCOMYCIN AGAINST *Staphylococcus aureus***

**PIRACICABA**

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Odontologia, área de Farmacologia, Anestesiologia e Terapêutica.

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Orientadora: Profa. Dra. Karina Cogo Müller

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Este exemplar corresponde à versão final da tese defendida pela aluna Andréia Borges Scriboni e orientada pelo Profa. Dra. Karina Cogo Müller

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A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

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## RESUMO

A vancomicina (VAN) é um antibiótico glicopeptídeo utilizado como primeira escolha no tratamento de infecções persistentes e recorrentes causadas por *Staphylococcus aureus* resistentes à meticilina (MRSA). No entanto, sua eficácia terapêutica é limitada devido às características físico-químicas da molécula, que dificultam a penetração no biofilme bacteriano. Assim, os objetivos deste estudo foram desenvolver e caracterizar vesículas unilamelares pequenas contendo VAN, de lipossomas convencionais (SUV VAN), lipossomas fusogênicos (SUV<sub>fuso</sub> VAN) e catiônicos (SUV<sub>cat</sub> VAN) e avaliar *in vitro* a atividade antimicrobiana dessas formulações sobre o biofilme produzido por *Staphylococcus aureus* (*S. aureus*) em comparação com a solução de VAN livre. Todas as formulações lipossomais na ausência (controle) e presença da VAN a 10 mg/mL foram preparadas, respectivamente, com fosfatidilcolina de ovo:colesterol:alfa-tocoferol (4:3:0,07 mol%), dioleoilfosfatidiletanolamina: dipalmitoilfosfatidilcolina:colesterol hemisuccinato:alfa-tocoferol (4:2:4:0,07 mol%) e fosfatidilcolina de ovo:estearilamina:colesterol:alfa-tocoferol (1:0,5:0,5:0,07 mol%). Os lipossomas obtidos foram caracterizados em termos de tamanho, índice de polidispersão (PDI), potencial zeta, morfologia por microscopia eletrônica de transmissão (MET), eficiência de encapsulação (%EE) e cinética de liberação *in vitro*. Para avaliação da eficácia antimicrobiana foram realizados ensaios de Concentração Inibitória Mínima (CIM), inibição da formação de biofilme e o teste de viabilidade celular em biofilme maduro de *S. aureus*, utilizando cepas de *S. aureus* meticilina sensível (MSSA) ATCC 29213 e MRSA ATCC 43300. SUV VAN mostraram melhor %EE (32,46%) e liberação sustentada (12 h) em relação às SUV<sub>fuso</sub> VAN, SUV<sub>cat</sub> VAN e VAN livre. A CIM, das formulações lipossomais e da VAN livre variou entre 1.56 a 0.78 µg/mL, para ambas as cepas testadas. As formulações lipossomais promoveram inibição da formação de biofilme de forma semelhante à VAN livre ( $p>0,05$ , ANOVA Tukey). No entanto, a VAN encapsulada mostrou maior eficácia antimicrobiana que a VAN livre na redução da viabilidade de biofilme já formado, sendo as formulações SUV<sub>fuso</sub> VAN e SUV<sub>cat</sub> VAN superiores à SUV VAN (ANOVA, Tukey,  $p<0,05$ ). A VAN encapsulada em SUV<sub>fuso</sub> e SUV<sub>cat</sub> aumentaram a atividade antimicrobiana contra biofilme de *S. aureus*, mostrando que essas formulações podem ser promissoras no controle de infecções causadas por esse microrganismo.

**Palavras-chave:** *Staphylococcus aureus*, Biofilme; Lipossomas fusogênicos; Lipossomas catiônicos; Vancomicina.

## ABSTRACT

Vancomycin (VAN) is a first-choice glycopeptide antibiotic for the treatment of persistent and recurrent infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). Nevertheless, its therapeutic efficacy is limited due to drug physicochemical characteristics, which make bacterial biofilms difficult to penetrate. In this study, we developed and characterized small unilamellar vesicles of conventional (SUV VAN), fusogenic (SUV<sub>fuso</sub> VAN), and cationic (SUV<sub>cat</sub> VAN) liposomes containing hydrochloride VAN. The *in vitro* antimicrobial activity of these formulations on *Staphylococcus aureus* (*S. aureus*) biofilms was further determined and compared with that of a free VAN solution. SUV, SUV<sub>fuso</sub> and SUV<sub>cat</sub> liposomes were characterized in terms of size, Polydispersity Index (PDI), zeta potential, morphology by Transmission Electron Microscopy (TEM), encapsulation efficiency (%EE), and *in vitro* release kinetics. Then the formulations were tested for their Minimum Inhibitory Concentration (MIC) and inhibitory activity on biofilm formation and viability, using methicillin-susceptible *S. aureus* (MSSA) ATCC 29213 and MRSA ATCC 43300 strains. SUV VAN showed better %EE (32.46%) and sustained release (12 h) than SUV<sub>fuso</sub> VAN, SUV<sub>cat</sub> VAN, and free VAN. The MIC values of liposomal formulations and free VAN ranged between 0.78 and 1.56 µg/mL against both strains, with no difference in inhibition of biofilm formation as compared to free VAN ( $P > 0.05$ , ANOVA with Tukey's post-hoc). Nevertheless, encapsulated VAN was found to have better antimicrobial efficacy than free VAN on the viability of preformed biofilms, being SUV<sub>fuso</sub> and SUV<sub>cat</sub> more active than SUV ( $P < 0.05$ , ANOVA with Tukey's post-hoc). In conclusion, we demonstrated the successful development and characterization of SUV, SUV<sub>fuso</sub> and SUV<sub>cat</sub> encapsulated VAN formulations with enhanced antimicrobial activity against mature *S. aureus* biofilm. Our findings indicate that these formulations may be promising candidates for *S. aureus* infection control.

**Key-words:** *Staphylococcus aureus*; Biofilm; Fusogenic liposomes; Cationic liposomes; Vancomycin.

## LISTA DE ABREVIATURAS E SIGLAS

CHEMS	Colesterol hemisucinato
CIM	Concentração Inibitória Mínima
Col	Colesterol
DCP	Dietilfosfato
DOPE	Dioleilfosfatidiletanolamina
DPPC	Dipalmitoilfosfatidilcolina
DSPC	Distearoilglicerofosfocolina
EPC	Fosfatidilcolina de ovo
MET	Microscopia Eletrônica de Transmissão
MLV	Vesículas Multilamelares
MRSA	<i>Staphylococcus aureus</i> resistentes à meticilina
MSSA	<i>Staphylococcus aureus</i> meticilina sensível
PDI	Índice de polidispersão
PE	Fosfatidiletanolamina
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
Sa	Estearilamina
SUV	Lipossoma Convencional
SUV VAN	Lipossoma convencional contendo Cloridrato de Vancomicina
SUV <sub>fuso</sub>	Lipossoma Fusogênico
SUV <sub>fuso</sub> VAN	Lipossoma Fusogênico contendo Cloridrato de Vancomicina
SUV <sub>cat</sub>	Lipossoma Catiônico
SUV <sub>cat</sub> VAN	Lipossoma Catiônico contendo Cloridrato de Vancomicina
VAN	Cloridrato de Vancomicina
VAN livre	Solução de Cloridrato de Vancomicina
$\alpha$ -T	Alfa-tocoferol
%EE	Eficiência de Encapsulação

## LIST OF ABBREVIATIONS AND ACRONYMS

CHEMS	Cholesterol hemisuccinate
Chol	Cholesterol
DCP	Dicethylphosphate
DOPE	Dioleoylphosphatidylethanolamine
DPPC	Dipalmitoylphosphatidylcholine
DSPC	Distearoylglycerophosphocholine
EPC	Egg phosphatidylcholine
MIC	Minimum Inhibitory Concentration
MLV	Multilamellar vesicles
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin-susceptible <i>Staphylococcus aureus</i>
PDI	Polydispersity Index
PE	Fosfatidiletanolamina
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
Sa	Stearylamine
SUV	Conventional Liposome
SUV VAN	Conventional Liposome containing Hydrochloride Vancomycin
SUV <sub>fuso</sub>	Fusogenic Liposome
SUV <sub>fuso</sub> VAN	Fusogenic Liposome containing Hydrochloride Vancomycin
SUV <sub>cat</sub>	Cationic Liposome
SUV <sub>cat</sub> VAN	Cationic Liposome containing Hydrochloride Vancomycin
TEM	Transmission Electron Microscopy
VAN	Hydrochloride Vancomycin
VAN livre	Hydrochloride Vancomycin Solution
$\alpha$ -T	Alpha-tocopherol
%EE	Encapsulation Efficiency

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## 1 INTRODUÇÃO

*Staphylococcus aureus* (*S. aureus*) é considerado um dos principais patógenos causadores de infecções nosocomiais fatais, bem como, as infecções adquiridas na comunidade, representando um grande desafio clínico na saúde pública mundial (Huang et al., 2011; McCarthy et al., 2015). Essa bactéria é responsável por uma ampla variedade de infecções clínicas, entre elas a endocardite infecciosa, infecções osteoarticulares, pleuropulmonar, além das infecções graves relacionadas aos dispositivos hospitalares, como próteses e cateteres (Holland et al., 2014; Tong et al., 2015). *S. aureus* também pode ser responsável por intoxicação alimentar (Peacock and Paterson, 2015). A mortalidade dos pacientes com bacteremias causadas por *S. aureus* supera 80% (Holland et al., 2014; Peacock and Paterson, 2015).

*S. aureus* é uma bactéria Gram positiva presente na microbiota anfibiônica da pele e superfícies de mucosa como do nariz, garganta, vagina, trato gastrointestinal (Liu GY, 2009; Foster et al., 2014). Essa bactéria produz na maioria das vezes infecções oportunistas graves, cuja gravidade pode variar de acordo com a resposta imunológica do paciente e bem como com o perfil de virulência da cepa bacteriana causadora da infecção (Ng et al., 2011). Esse microrganismo apresenta a capacidade de produzir vários fatores de virulência como exopolissacarídeos, exoproteínas, ácido teicóico, proteína A, catalase, coagulase, fibrinolisa e as toxinas estafilocócicas (Mishra et al., 2012; Peacock and Paterson, 2015), que sobretudo interferem na interação bacteriana com o hospedeiro ao longo do processo de colonização, nas estratégias de evasão das defesas do organismo e na inflexão da resposta imune (Foster et al., 2014). Uma vez que *S. aureus* invade a corrente sanguínea, por obstrução epitelial, o mesmo encontra um ambiente favorável para o crescimento das suas células planctônicas e condições positivas para a produção dos fatores de aderência. Assim, essas bactérias quando alcançam o interior do organismo podem ser removidas pelas células do sistema imunológico do hospedeiro ou iniciam a formação do biofilme utilizando as proteínas da matriz extracelular como mecanismo para fixação nas superfícies orgânicas (Archer et al., 2011). Ainda, essa capacidade de formar biofilmes é fundamental para colonização

em superfícies inorgânicas, como os cateteres entre outros dispositivos médicos que provavelmente são responsáveis por infecções nosocomiais (Foster et al., 2014).

Os biofilmes são agregados de microcolônias bacterianas aderidas umas às outras formando comunidades complexas envolvidas por uma matriz extracelular que se fixam em superfícies bióticas ou abióticas (Islam et al., 2014). O mecanismo de formação do biofilme envolve 4 etapas sequenciais fundamentais como, a adesão na superfície, produção da matriz extracelular, formação de colônias e dispersão do biofilme (Abdalla et al., 2014). A matriz extracelular é constituída por proteínas, ácidos nucleicos, lipídeos e principalmente por polissacarídeos e é responsável pela maturação do biofilme, permitindo a adesão primária dos microrganismos, colaborando para a estrutura do biofilme e propiciando ampla proteção em relação a resposta imunológica do hospedeiro e à ação dos antimicrobianos (Renner e Weibel, 2011). Os dispositivos médicos implantáveis são suscetíveis à colonização por *S. aureus* e as infecções causadas por seu biofilme têm sido associadas com dispositivos que vão desde cateteres implantados para próteses valvulares cardíacas, estimuladores cardíacos, lentes de contato, shunts fluido cefalorraquidiano, próteses e linhas intravasculares (McCarthy et al., 2015).

Durante as últimas décadas, *S. aureus* passou por várias fases de resistência a antibióticos e atualmente é resistente a maioria das classes de antimicrobianos, como penicilinas, cefalosporinas, macrolídeos, aminoglicosídeos, cloranfenicol, tetraciclina, tornando cada vez mais obscuro o tratamento e controle das infecções (Gill et al., 2005; Huang et al., 2011). A resistência atribuída às penicilinas e aos beta-lactâmicos como meticilina e oxacilina, foram relatadas em 1960 com o aparecimento das cepas *S. aureus* resistentes à meticilina (MRSA). MRSA se caracteriza pela presença do gene *mecA* ou outro mecanismo de resistência à meticilina, como redução significativa da afinidade das Proteínas Ligadoras de Penicilina (penicillin-binding protein, PBP) pela meticilina (Huang et al., 2011; CLSI, 2012; Peacock and Paterson, 2015). Esta resistência permite a biossíntese da parede celular, o alvo de beta-lactâmicos, mesmo na presença de concentrações inibitórias do antibiótico (Peacock and Paterson, 2015). O aparecimento de infecções causadas por MRSA na comunidade (CAMRSA) sugere uma maior patogenicidade dessa bactéria, que pode estar associado com novos elementos genéticos (Foster et al., 2014). Diante da elevada virulência, o tratamento



das infecções causadas por MRSA se estreitou na utilização da vancomicina como um dos últimos recursos para o tratamento (Pumerantz et al., 2011; Ng et al., 2011; Elkhodairy et al., 2014; Holland et al., 2014; Honary et al., 2014; Men et al., 2016).

A vancomicina (VAN) é um antibiótico da classe dos glicopeptídeos que possui elevada eficiência terapêutica nas infecções causadas por bactérias Gram-positivas (Howden et al., 2010). A molécula da VAN foi isolada primeiramente em 1956 a partir da fermentação de culturas de *Amycolatopsis orientalis* (anteriormente *Streptomyces orientalis*) pelo Laboratório Lilly (Indianapolis, IN, USA) (Butler et al., 2014). Em 1958, foi introduzida pela primeira vez na clínica médica (Vila et al., 2007). A estrutura da VAN foi definida no final dos anos 70 como sendo uma molécula tricíclica de elevada hidrofiliabilidade, alto peso molecular (1449,2 g mol<sup>-1</sup>) e com vários grupamentos catiônicos e aniônicos (Enrique et al., 2008; Howden et al., 2010; Ng et al., 2011). O mecanismo de ação da VAN ocorre por meio de ligações de hidrogênio com a fração proteica terminal (L-lisina-D-alanil-D-alanina) dos monômeros de mureína responsáveis pela biossíntese da parede celular bacteriana (Howden et al., 2010). Em *S. aureus*, o primeiro de alvo da VAN está na parede celular onde se liga ao resíduo D-alanil-D-alanina nas camadas de peptidoglicanos finalizados. Posteriormente, o segundo alvo localiza-se na membrana citoplasmática, nos monômeros mureína utilizados como substrato para a glicosiltransferase na produção dos peptidoglicanos (Howden et al., 2010; Chakraborty et al., 2012). A ligação da VAN nos peptidoglicanos já formados na parede celular não inibe a biossíntese de peptidoglicano, apenas pode interferir nas ligações cruzadas entre as cadeias de mureína (Howden et al., 2010; Butler et al., 2014). Assim, A VAN deve se ligar aos monômeros de mureína situadas na membrana citoplasmática para inibir completamente a biossíntese do peptidoglicano e portanto, concluir seu efeito antibacteriano (Howden et al., 2010; Butler et al., 2014; Elkhodairy et al., 2014).

Ainda que a VAN seja uma das poucas opções para o tratamento de infecções causadas por MRSA, sua terapêutica vem acompanhada de várias limitações referentes as suas características físico-químicas (Nicolosi et al., 2010). Sua eficácia terapêutica é restrita em consequência das suas propriedades farmacocinéticas desfavorecidas, incluindo um tempo curto de meia-vida, na faixa de 4 – 11 horas, além de um elevado peso molecular e alta hidrossolubilidade o que dificultam sua penetração nos tecidos, bem como, no biofilme bacteriano (Nicolosi et

al., 2010; Butler et al., 2014; Moghadas-Sharif et al., 2015). Assim, a maior concentração da VAN permanece no plasma, de onde rapidamente é eliminada pelos rins e por fagócitos (Muppidi et al., 2011). A terapêutica com VAN apresenta várias reações adversas, como diarreia aquosa grave, insuficiência renal e a reação de hipersensibilidade conhecida como "síndrome do pescoço vermelho" são dependentes da dose, comumente, ocorre com doses elevadas de VAN, administradas em caso de infecções severas com a finalidade de melhorar a eficácia terapêutica (Pumerantz et al., 2011; Rose et al., 2012; Honary et al., 2014). Ainda, outras reações adversas são alusivas ao uso da VAN, incluindo a ototoxicidade, neutropenia, febre, anafilaxia, trombocitopenia e flebite (McAuley, 2012).

As falhas clínicas e elevada toxicidade da VAN, em particular o fato que a dose terapêutica é muito próxima da dose tóxica, desencadeou interesse de alguns estudos para, principalmente, aumentar sua eficácia antimicrobiana e ao mesmo tempo, reduzir seus efeitos tóxicos ao paciente (Forier et al., 2014; Honary et al., 2014). A utilização da nanotecnologia na elaboração de formulações de medicamentos antimicrobianos tem sido explorada como um mecanismo promissor para elevar a eficácia terapêutica de antibióticos e até por vezes, superar a resistência bacteriana (Drulis-Kawa and Dorotkiewicz-Jach, 2010; Honary et al., 2014). A liberação de antibióticos encapsulados em lipossomas proporciona muitas vantagens, incluindo a distribuição controlável e possivelmente uniforme para o tecido alvo, aumento das interações com as bactérias do biofilme e células do hospedeiro, aumento da meia-vida do fármaco e o tempo de circulação sistêmica, redução do número de doses administradas redução dos efeitos adversos (Drulis-Kawa et al., 2009; Pumerantz et al., 2011; Sande et al., 2012; Honary et al., 2014; Srinivas et al., 2015).

Lipossomas são vesículas lipídicas esféricas nanométricas preparadas a partir da dispersão de lipídeos em água, descritos pela primeira vez por Bangham na década de 60 (Banerjee, 2001). Como a composição das vesículas é semelhante com as membranas biológicas, os lipossomas são biocompatíveis e biodegradáveis (Malinovsky et al., 1997; Grant, 2002). Os lipossomas podem ser classificados quanto ao tamanho e ao número de bicamadas lipídicas, podendo ser nomeadas de vesículas multilamelares (MLV – de 500 a 5000 nm com várias bicamadas), vesículas unilamelares grande (LUV – de 200 a 800 nm com uma bicamada) e

vesículas unilamelares pequenas (SUV – em torno de 100 nm com uma bicamada) (Torchilin, 2005; de Paula et al., 2012). A encapsulação de um fármaco em lipossomas depende da sua hidrofiliabilidade ou lipofiliabilidade, sendo que os fármacos polares permanecem na fase aquosa (no núcleo) ou entre as bicamadas lipídicas, enquanto os fármacos apolares permanecem no interior da bicamada lipídica (Grant, 2002). As características dos lipossomas como, tamanho, carga de superfície e funcionalidades podem ser simplesmente alteradas através da combinação de novos componentes à mistura de lipídeos antes da preparação ou pelo tipo de método de preparação dos mesmos (Torchilin, 2005).

Diferentes lipossomas com diferentes propriedades físico-químicas estão sendo desenvolvidos com objetivo de melhorar a penetração de fármacos nas células-alvo, entre eles as vesículas fusogênicas e catiônicas (Kim et al., 1999; Nicolosi et al., 2015). Os lipossomas fusogênicos ou lipossomas pH-sensíveis são vesículas compostas por fosfolipídeos especiais que facilitam sua fusão com as células-alvo promovendo a liberação do fármaco encapsulado no interior das mesmas (Nicolosi et al., 2010). O estudo destes lipossomas foi proposto para liberação de fármacos em tecidos onde o pH está alterado como infecções, inflamações e tumores (Aoki et al., 2015). Os lipossomas fusogênicos são compostos de lipídeos derivados da fosfatidiletanolamina (PE), como a dioleilfosfatidiletanolamina (DOPE) que na presença de água apresenta organização na forma hexagonal e assim não possui a capacidade de formar lipossomas (Aoki et al., 2015). Para formar lipossomas com DOPE é preciso adicionar lipídio carboxilado para estabilizar a formulação, como o hemisuccinato de colesterol (CHEMS – colesterol hemi-succinato), mantendo a forma ionizada negativa no pH fisiológico (Aoki et al., 2015). O DOPE e CHEMS se ligam ao fosfolipídio e assim surgem as repulsões eletrostáticas entre a carboxila do CHEMS e os grupos amins do fosfolipídeos possibilitando a organização lamelar e formando os lipossomas. As vesículas fusogênicas são captadas pelas células por endocitose, ocorrendo a desestabilização e a fusão com a membrana dos endossomas provocando assim a liberação do fármaco encapsulado no citoplasma da célula hospedeira (Nicolosi et al., 2010; Zhang et al., 2014; Aoki et al., 2015). A fusão das vesículas fusogênicas com a membrana endossomal impedirá a fusão dos endossomas com o fármaco encapsulado, assim os lipossomas fusogênicos protegem a degradação do fármaco

pelas enzimas presentes neste último e garante maior concentração de fármaco no interior das células alvos (Hiraka et al., 2008; Nicolosi et al., 2015).

Lipossomas catiônicos são ideais para interação eletrostática com a carga negativa presente na superfície das células (Soema et al., 2015). A eficácia dos lipossomas catiônicos está relacionada com a adsorção da vesícula na parede celular, por meio de interações eletrostáticas, que posteriormente podem se difundir alcançando o interior das células (Kim et al., 1999). Os lipídeos mais comumente utilizados nestes lipossomas são, estearilamina (Sa), brometo de dimetildioctadecilamônio (DDBA), dimetilaminoetano carbamoil colesterol (DC-col), Dioleoiltrimetilamôniopropano (DOTAP), dioleilepoxipropiltrimetilamônio (DOTMA) e brometo dedioctadecildimetilamônio (DODAB) (Bozzuto e Molinari, 2015).

Já foi demonstrado que os lipossomas apresentam capacidade de concentrar antimicrobianos, como a VAN nas interfaces do biofilme de *S. aureus*, melhorando sua atividade antimicrobiana (Kim et al., 1999). Estudos *in vitro* mostraram menores valores da Concentração Inibitória Mínima (CIM) para VAN encapsulada em diferentes formulações lipossomais, com destaque para os lipossomas catiônicos contra *S. aureus* de isolado clínico e MRSA (Kadry et al., 2004, Sande et al., 2012). Em um estudo *in vivo*, utilizando modelos de infecção óssea por *S. aureus* de isolado clínico em coelhos, foi demonstrado melhor eficiência terapêutica da VAN encapsulada em lipossomas catiônicos em comparação com a VAN livre (Kadry et al., 2004). Estes autores ainda revelaram que a encapsulação da VAN apresentou reduzida nefrotoxicidade (Kadry et al., 2004). Outro estudo *in vitro* em cultura de macrófagos revelou que a VAN encapsulada em lipossoma convencional é capaz de atingir maior concentração no interior dos macrófagos infectados aumentando a eliminação de MRSA (Pumerantz et al., 2011). Também foi demonstrado em lipossoma fusogênico composto de dioleoilfosfatidiletanolamina:dipalmitoilfosfatidilcolina:colesterol hemisuccinato (DOPE:DPPC:CHEMS – 4:2:4:0,07 mol%) reduziu a CIM contra isolados clínicos de *Escherichia coli* e *Acinetobacter baumannii* (bactérias Gram negativas) (Nicolosi et al., 2010).

A necessidade de melhorar a eficiência antimicrobiana da VAN e assim proporcionar melhores resultados clínicos na terapêutica de infecções severas causadas por cepas de *S. aureus* foram as principais motivações para este estudo.

Por isso, o presente trabalho teve como objetivo desenvolver e caracterizar SUV, SUV<sub>fuso</sub> e SUV<sub>cat</sub> contendo VAN, além de, avaliar a atividade antimicrobiana dessas formulações em biofilme de *S. aureus*, comparando com a VAN livre em solução.

A presente tese apresentada está em formato alternativo, de acordo com a Normalização de Teses e Dissertações da FOP/UNICAMP, Norma da CCPG/001/2015.

## **2 ARTICLE: Characterization and antibiofilm activity of liposome-encapsulated vancomycin against *Staphylococcus aureus*.**

Scriboni AB, Couto VM, Freires IA, Groppo FC, de Paula E, Franz-Montan M, Cogo-Müller K.

### **Abstract**

Vancomycin (VAN) is a first-choice glycopeptide antibiotic for the treatment of persistent and recurrent infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). Nevertheless, its therapeutic efficacy is limited due to drug physicochemical characteristics, which make bacterial biofilms difficult to penetrate. In this study, we developed and characterized small unilamellar vesicles of conventional (SUV VAN), fusogenic (SUV<sub>fuso</sub> VAN), and cationic (SUV<sub>cat</sub> VAN) liposomes containing hydrochloride VAN. The *in vitro* antimicrobial activity of these formulations on *Staphylococcus aureus* (*S. aureus*) biofilms was further determined and compared with that of a free VAN solution. SUV, SUV<sub>fuso</sub> and SUV<sub>cat</sub> liposomes were characterized in terms of size, Polydispersity Index (PDI), zeta potential, morphology by Transmission Electron Microscopy (TEM), encapsulation efficiency (%EE), and *in vitro* release kinetics. Then the formulations were tested for their Minimum Inhibitory Concentration (MIC) and inhibitory activity on biofilm formation and viability, using methicillin-susceptible *S. aureus* (MSSA) ATCC 29213 and methicillin-resistant *S. aureus* (MRSA) ATCC 43300 strains. SUV VAN showed better %EE (32.46%) and sustained release (12 h) than SUV<sub>fuso</sub> VAN, SUV<sub>cat</sub> VAN, and free VAN. The MIC values of liposomal formulations and free VAN ranged between 0.78 and 1.56 µg/mL against both strains, with no difference in inhibition of biofilm formation as compared to free VAN ( $P > 0.05$ , ANOVA with Tukey's post-hoc). Nevertheless, encapsulated VAN was found to have better antimicrobial efficacy than free VAN on the viability of preformed biofilms, being SUV<sub>fuso</sub> and SUV<sub>cat</sub> more active than SUV ( $P < 0.05$ , ANOVA with Tukey's post-hoc). In conclusion, we demonstrated the successful development and characterization of SUV, SUV<sub>fuso</sub> and SUV<sub>cat</sub> encapsulated VAN formulations with enhanced antimicrobial activity against mature *S. aureus* biofilm. Our findings indicate that these formulations may be promising candidates for *S. aureus* infection control.

**Key-words:** *Staphylococcus aureus*; Biofilm; Fusogenic liposomes; Cationic liposomes; Vancomycin.

## Introduction

*S. aureus* is a Gram-positive bacterium responsible for the majority of nosocomial and community-acquired infections. Notably, *S. aureus* infections remain a global public health issue highly costly for the healthcare system, with increasing morbidity and mortality rates worldwide (Chakraborty et al., 2010; Elkhodairy et al., 2014; Honary et al., 2014; Holland et al., 2014). Among several virulence traits, *S. aureus* has the ability to invade and survive within phagocytic cells, escape from immune response, and form robust biofilms on organic and inorganic surfaces, such as mucous membranes and catheters or other medical devices, respectively (Muppidi et al., 2011; Pumerantz et al., 2011; Archer et al., 2011). Today, over 90% of *S. aureus* strains are found to be resistant to methicillin, penicillin, aminoglycosides, macrolides, lincosamides, and other beta-lactams (Muppidi et al., 2011; Sande et al., 2012; Chakraborty et al., 2012; Elkhodairy et al., 2014; Shi et al., 2014).

In this scenario of microbial resistance, vancomycin (VAN) is considered a first-choice antibiotic for the treatment of methicillin-resistant *S. aureus* (MRSA) infections (Ng et al., 2011; Pumerantz et al., 2011; Elkhodairy et al., 2014; Holland et al., 2014; Honary et al., 2014; Men et al., 2016). VAN is an antibacterial glycopeptide which inhibits cell wall peptidoglycan biosynthesis in Gram-positive bacteria (Howden et al., 2010). While VAN remains a first-choice antibiotic for the treatment of MRSA infections, its therapeutic efficacy is limited due to its high molecular weight (1449.2 g mol<sup>-1</sup>) and high hydrophilicity, which restricts drug interaction with bacterial cells and hinders penetration into biofilms (Howden et al., 2010; Nicolosi et al., 2010; Butler et al., 2014; Moghadas-Sharif et al., 2015). VAN toxicity towards host tissues may be another limiting factor, as there are reports of side effects upon its use, including severe watery diarrhea, kidney failure (Pumerantz et al., 2011; Rose et al., 2012; Honary et al., 2014; Zhang et al., 2014), ototoxicity, neutropenia, fever, anaphylaxis, thrombocytopenia, and phlebitis (McAuley, 2012).

Bacterial biofilms are characterized by aggregation of specific bacterial species adhered to a substrate forming highly organized microbial communities (McCarthy et al., 2015). Biofilm-forming bacteria display a differentiated phenotype



compared to planktonic cells and have the ability to produce an extracellular polymeric matrix composed mainly of polysaccharides (Khameneh et al., 2014; Dong et al., 2015; McCarthy et al., 2015). This scaffold provides an extremely robust defense mechanism, which hinders antibiotic penetration into the biofilm structure and thus substantially reduces bacterial susceptibility (Howden et al., 2010; Khameneh et al., 2014; Shi et al., 2014; Dong et al., 2015; McCarthy et al., 2015; Moghadas-Sharif et al., 2015).

The shortcomings of traditional treatment with VAN, increased microbial resistance, and difficulty to treat biofilms have encouraged the development of drug-carrier systems, such as liposome-encapsulated VAN (Kadry et al., 2004; Drulis-Kawa et al., 2009; Nicolosi et al., 2010; Muppidi et al., 2011; Pumerantz et al., 2011; Sande et al., 2012; Srinivas et al., 2015). It has been shown that liposomal sustained release of VAN (i) enhances antibacterial efficacy, due to higher interaction of the antibiotic molecule with bacterial cells (Kim et al., 1999); (ii) improves pharmacokinetics (Ma et al., 2011); (iii) reduces toxicity (Sande et al., 2012); and (iv) increases the antimicrobial spectrum of action against Gram-negative bacteria (Nicolosi et al., 2010). Furthermore, liposomes can facilitate antibiotic penetration into bacterial cells and therefore increase drug concentration in the inner layers of the biofilm (Moghadas-Sharif et al., 2015).

Liposomes are nanometric, amphiphilic vesicles composed of a variety of phospholipids arranged in lipid bilayers with an aqueous core, which allows encapsulation of lipophilic or hydrophilic drugs without chemical alteration of their molecules (Malinovsky et al., 1997; Grant, 2002). Liposome composition can be specifically modulated to favor adsorption onto, or fusion through, microbial cell membrane. Likewise, vesicle surfaces can be changed based on the characteristics of the infectious agent (Nicolosi et al., 2010). Among some types of liposomes with the ability of interacting with bacterial biofilm cells are fusogenic and cationic liposomes (Kim et al., 1999; Nicolosi et al., 2010).

Fusogenic liposomes are phospholipid vesicles that may fuse with biological membranes, thereby increasing drug contact and delivery into cells. They consist of lipids, such as dioleoyl-phosphatidylethanolamine (DOPE) and cholesterol hemisuccinate (CHEMS), which provide increased fluidity to the lipid bilayer and may destabilize biological membranes (Nicolosi et al., 2010; Aoki et al., 2015; Nicolosi et



al., 2015). These lipids assume the liquid crystalline state under specific chemical conditions, e.g., acidic milieu or in the presence of cations (Fourier et al., 2014).

Cationic liposomes are composed of lipids with a positive residual charge, such as stearylamine (SA), dimethyldioctadecylammonium bromide (DDBA), dimethylaminoethane carbamoyl cholesterol (DC-chol), and dioleoyltrimethylammoniumpropane (DOTAP), which can facilitate electrostatic interactions with bacterial cell wall and biofilms, both negatively charged (Kim et al., 1999; Torchilin, 2012; Zhang et al, 2014; Moghadas-Sharif et al., 2015).

While fusogenic and cationic liposomes have proven advantages in interacting with bacterial cells and formed biofilms, there is no consensus in the literature on the ideal composition of liposome-encapsulated VAN formulations able to enhance drug delivery and increase their antimicrobial power. Thus, in the present study we developed and characterized small unilamellar vesicles of conventional (SUV VAN), fusogenic (SUV<sub>fuso</sub> VAN), and cationic (SUV<sub>cat</sub> VAN) liposomes containing hydrochloride VAN. We further determined the *in vitro* antimicrobial activity of these formulations on *S. aureus* biofilms and compared with that of a free VAN solution.

## **Material and Methods**

### **Materials**

VAN hydrochloride was kindly provided by Teuto/Pfizer Laboratory (Anápolis, GO, Brazil). HEPES buffer, cholesterol (Chol), alpha-tocopherol ( $\alpha$ -T) and egg phosphatidylcholine (EPC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloroform used for dilution of lipids was obtained from Merck (Darmstadt, Germany). Dioleoylphosphatidylethanolamine (DOPE), dipalmitoylphosphatidylcholine (DPPC), cholesterol hemisuccinate (CHEMS) and stearylamine (Sa) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL USA.).

### **Preparation of liposomal formulations**

SUV VAN, SUV<sub>fuso</sub> VAN, SUV<sub>cat</sub> VAN were prepared containing VAN at 10 mg/mL. Plain, VAN-free formulations were used as negative controls in the experiments (SUV, SUV<sub>fuso</sub>, and SUV<sub>cat</sub>). All liposomal formulations were prepared to be at 10 mM lipid concentration with the following composition: SUV – EPC:Chol: $\alpha$ -T

(4:3:0.07, mol%) (adapted from Cereda et al., 2006); SUV<sub>fuso</sub> – DOPE:DPPC:CHEMS:α-T (4:2:4:0.07, mol%) (adapted from Nicolosi et al., 2010); SUV<sub>cat</sub> – EPC:Sa:Chol:α-T (1:0.5:0.5:0.07, mol%) (adapted from Kadry et al., 2004), respectively. All formulations were prepared in HEPES buffer (80 mM) containing 150 mM NaCl (pH 7.4).

Preparation of liposomal formulations was carried out as previously described, with modifications (Cereda et al., 2006). Briefly, the lipids were dissolved in chloroform, evaporated under nitrogen flow to obtain the lipid film, and vacuumed for 2 h to ensure complete solvent removal. Subsequently, the film was hydrated in HEPES buffer with or without VAN hydrochloride solution. Then the suspension was vortexed for 5 min to form large multilamellar vesicles (MLVs). The suspensions were extruded under nitrogen flow at high pressure (Extruder Emulsiflex C5, Avestin, Inc., Ottawa, ON, Canada) 12 times using polycarbonate membrane initially with 400 nm pores and then with 100 nm pores so that to obtain small unilamellar vesicles. The extrusion of SUV<sub>fuso</sub> formulation was performed in water bath at 50°C, which is higher than the DPPC phase transition temperature (Nicolosi et al., 2010). All VAN-containing and VAN-free liposomal formulations were used fresh for the characterization and antimicrobial assays.

## **Characterization of liposomal formulations**

### *Morphological analysis*

The morphology of the different types of VAN-containing or plain liposomes was analyzed by Transmission Electron Microscopy (TEM) (906 LEO - ZEISS, Jena, Germany). Briefly, one drop of each formulation was added to a copper-coated grid with 200 mesh for 10 s (Electron Microscopy Sciences, Fort Washington, PA). Subsequently, uranyl acetate aqueous solution (2%, w/v) was added and kept at room temperature for 4 h. The readings were taken under 80 kV electron beams (Franz-Montan et al., 2015; da Silva et al., 2016).

### *Determination of size, polydispersity index and zeta potential*

Liposomal vesicles were diluted in deionized distilled water for evaluation of the average size (nm), polydispersity index (PDI), and zeta potential (mV) by the

dynamic light scattering method (dynamic light scattering - DLS) using Zeta-Sizer nanoseries Nano ZS equipment (Malvern Instruments Ltd., Worcestershire, UK, England) at 25°C in triplicate.

#### *Vancomycin Encapsulation Efficiency*

The encapsulation efficiency (%EE) of VAN into liposomal formulations was determined by ultrafiltration-centrifugation method (da Silva et al., 2016). Free, unencapsulated VAN was separated from encapsulated VAN by ultracentrifugation (Optima L-90K Ultracentrifuge, Beckman Coulter Inc. Pasadena, California, USA) at 120,000 g for 2 h at 10°C. Aliquots from the supernatant were diluted in deionized distilled water and analyzed spectrophotometrically at 280 nm (Varian Cary® 50 UV-Vis, Varian Inc., Palo Alto, CA, USA). The %EE was calculated based on the concentration of unencapsulated VAN over the concentration of VAN in solution, using the formula as follows:

$$\%EE = \frac{[\text{VAN solution}] - [\text{unencapsulated VAN}]}{[\text{VAN solution}]} \times 100$$

#### *Evaluation of vancomycin release in vitro*

The drug release assay was performed using the Franz vertical diffusion cell (Franz, 1975), which consists of two compartments – one donor and one receptor – separated by a regenerated cellulose membrane (Spectra/Por® 2) with molecular exclusion limit of 12000-14000 Da (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) (de Araújo et al., 2008; da Silva et al., 2016). An aliquot of 1 mL of the liposomal suspensions was added to the donor compartment, while the receptor compartment was filled with 4 mL of buffer (pH 7.4), maintained at 37 °C and 400 rpm agitation. Aliquots of the receptor medium were removed throughout the 12-hour experiment and analyzed by spectrophotometry at 280 nm (Varian Cary® 50 UV-Vis, Varian Inc., Palo Alto, CA, USA). The collected volume was replaced with fresh medium due to the dilution effect.

## Evaluation of Antimicrobial Activity

### *Microorganisms and Growth Conditions*

Methicillin-susceptible *S. aureus* (MSSA) ATCC 29213 and methicillin-resistant *S. aureus* (MRSA) ATCC 43300 strains were used in this study. Microorganisms were maintained in Tryptone Soy Broth (TSB) (Dfico®, New Jersey, USA) with 20% glycerol at -80°C, and cultivated onto Tryptone Soy Agar (Dfico®, New Jersey, USA) plates at 37°C. Mueller Hinton Broth (MHB) (Dfico®, New Jersey, USA) was used in the MIC assay, while Brain Heart Infusion (Dfico®, New Jersey, USA) plus 1% D-glucose (Sigma-Aldrich, St. Louis, MO, USA) was used in the biofilm killing assays.

### *Experimental Groups*

Test formulations consisted of VAN-containing and VAN-free SUV, SUV<sub>fuso</sub> and SUV<sub>cat</sub>. The experimental groups were set as follows: A – culture medium, test formulation and inoculum; B – culture medium, control formulation and inoculum; C – culture medium, free VAN solution and inoculum; D – culture medium, HEPES buffer (vehicle) and inoculum; E – culture medium and test formulation; F – culture medium and inoculum; and G – culture medium alone.

### *Minimum Inhibitory Concentration (MIC)*

The MIC was determined by the microdilution method, as previously described by the CLSI (CLSI, 2012). The formulations were added to 96-well microplates and serially diluted to obtain concentrations ranging from 0.025 to 50 µg/mL. Bacterial inoculum was prepared and adjusted in spectrophotometer ( $\lambda$  625nm, abs 0.1) to a final concentration of  $5 \times 10^4$  CFU/mL in the wells. The plates were incubated at 37°C for 24 h and the absorbance was read at 620 nm (Biochrom ASYS UVM 340, Biochrom, Cambridge, England). The MIC was defined as the lowest concentration of the formulation which inhibited visible bacterial growth. The assays were performed in six replicates.

### *Effects on Biofilm Formation*

The liposomal formulations were tested for their ability to inhibit biofilm formation and adherence according to the protocol proposed by Graziano et al.

(2015) and Wu et al. (2013). BHI medium plus 1% glucose, test formulations and *S. aureus* cell suspension (final concentration of  $5 \times 10^4$  CFU/mL) were added to 96-well U-bottom microplates. After 24 h incubation, the supernatant was removed and the wells were washed three times with distilled water to remove loosely bound or non-adhered cells. Biofilms were stained with 0.4% crystal violet, solubilized with 98% ethanol and read in a microplate reader at 575 nm (Asys UVM 340, Biochrom, Cambridge, England).

#### *Effects on Biofilm Viability*

The liposomal formulations were next tested for their inhibitory effects on biofilm viability, as previously described (Graziano et al., 2015). Cellulose acetate membranes (25 mm diameter, 0.2  $\mu$ m pores) (Sartorius Stedim GmbH, Guxhagen, Hessen, Germany) were used as substrates for *S. aureus* biofilm formation. The membranes were placed in 6-well plates containing BHI medium plus 1% glucose and bacterial suspension (approximately  $1 \times 10^6$  CFU/mL in each well). The plates were incubated at 37 °C for 24 h. Then the membranes were transferred to new plates containing fresh BHI plus 1% glucose, and biofilms were treated with the formulations at 1xMIC, 10xMIC, and 50xMIC for 24 h. Treated biofilm-coated membranes were gently washed with 0.9% NaCl, and sonicated and vortexed for 30 s (VibraCell400W, Sonics & Materials Inc., Newtown, CT, USA). Ten-microliter aliquots were collected from each tube, serially diluted, and plated for CFUs onto TSA. The plates were incubated at 37 °C for 24 h.

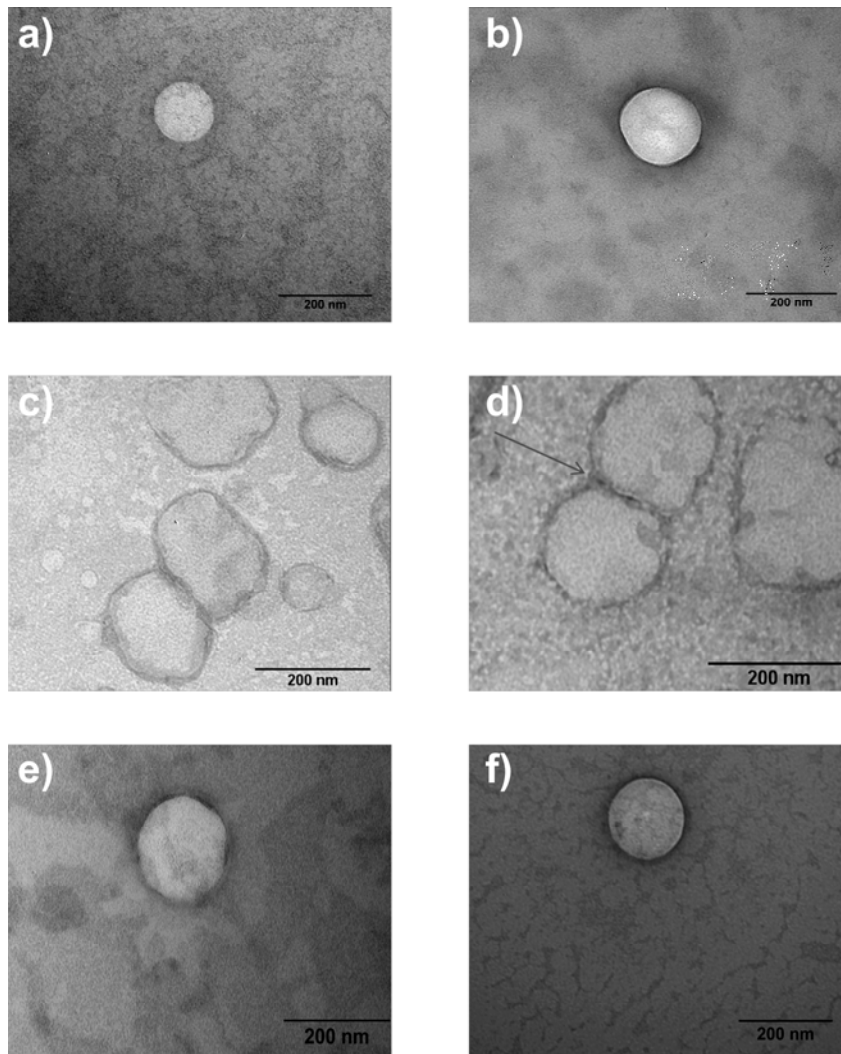
#### **Statistical analysis**

Data distribution was analyzed by Shapiro-Wilks test, and the findings related to characterization of liposomal formulations – size, PDI, zeta potential, and %EE – were compared using unpaired *t*-test. The data on *in vitro* release kinetics and biofilm assays were analyzed by two and one-way analysis of variance (ANOVA), respectively, followed by Tukey's post-hoc test. Statistical analyses were performed on Origin 8.0 (Microcal TM Software Inc., EUA) and GraphPad Prism 6.0 (San Diego, California, USA). The data were presented as mean and standard deviation (SD), with a 5% significance level.

## Results

### Characterization of liposomal formulations

TEM images confirmed that the liposomal vesicles had spherical shape and a single lipid bilayer. Vesicle size in all formulations ranged between 100 and 200 nm. Illustrative images of SUV, SUV<sub>fuso</sub>, and SUV<sub>cat</sub> are presented in **Figure 1**. As exemplified in Figure 1c and 1d, some vesicles were found to merge with each other, which typically characterizes this type of liposome.



**Figure 1.** Transmission electron microscopy (TEM) images of the liposomal SUVs developed in this study. The left panel represents plain vesicles and the right panel indicated VAN-containing vesicles as follows: (a, b) SUV; (c,d); SUV<sub>fuso</sub>; and (e, f) SUV<sub>cat</sub>. Bars indicate 200 nm, with 100kX magnification).

The means and standard deviations of size, polydispersity index (PDI), zeta potential and %EE of the liposomal formulations are presented in Table 1. There was a significant increase in the size and PDI values of VAN-containing formulations as compared to their plain controls ( $P<0.05$ ). Moreover, as expected, the analysis of zeta potential confirmed the presence of negative charges on SUV and SUV<sub>fuso</sub> liposomes and positive charges on SUV<sub>cat</sub>. VAN encapsulation decreased zeta potential module in SUV VAN and SUV<sub>fuso</sub> VAN liposomes ( $P<0.05$ ), while increased zeta potential in SUV<sub>cat</sub> VAN, as compared to their respective controls ( $P<0.05$ ). Higher %EE values were observed in SUV VAN, followed by SUV<sub>fuso</sub> VAN and SUV<sub>cat</sub> VAN.

**Table 1. Mean ( $\pm$ SD) of the size (nm), polydispersity index (PDI), zeta potential (mV) and encapsulation efficiency (%EE) of the liposomal formulations developed in this study.**

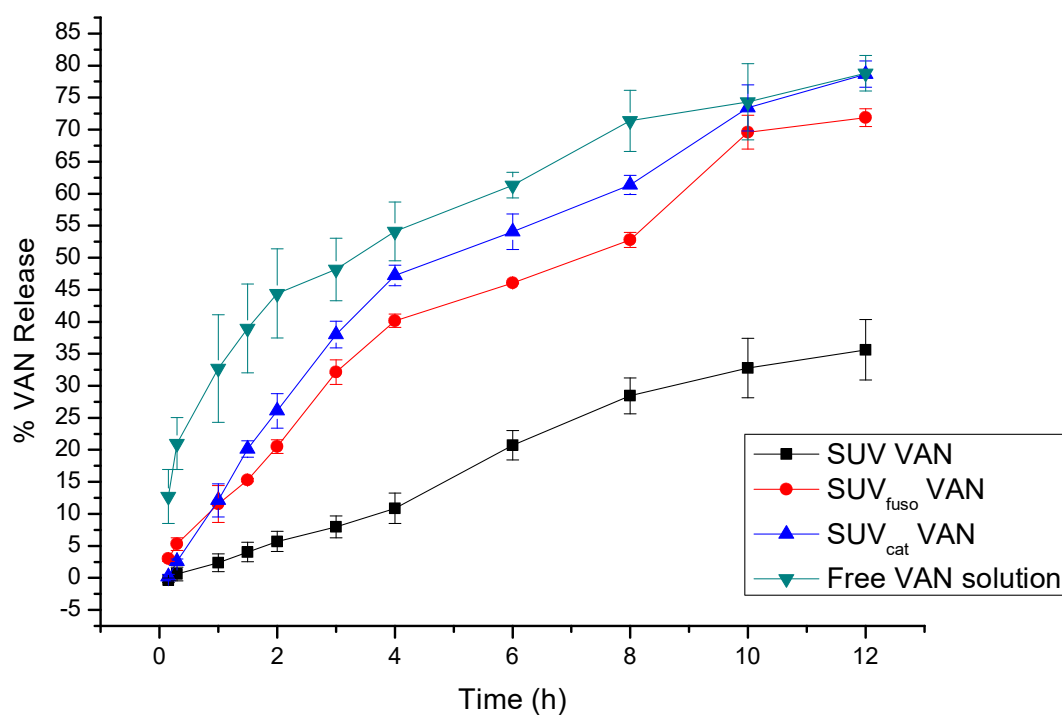
Formulation	Size (nm $\pm$ SD)	PDI ( $\pm$ SD)	Zeta Potential (mV $\pm$ SD)	% EE ( $\pm$ SD)
SUV	121.2 $\pm$ 0.41	0.06 $\pm$ 0.00	- 23.6 $\pm$ 1.9	-----
SUV VAN	152.6 $\pm$ 0.85 *	0.20 $\pm$ 0.00 *	-14.8 $\pm$ 0.3 *	32.46 $\pm$ 0.08
SUV <sub>fuso</sub>	103.7 $\pm$ 0.27	0.06 $\pm$ 0.00	- 48.6 $\pm$ 4.9	-----
SUV <sub>fuso</sub> VAN	134.3 $\pm$ 0.57 *	0.28 $\pm$ 0.01*	- 41.3 $\pm$ 2.3 *	11.44 $\pm$ 0.08
SUV <sub>cat</sub>	124.4 $\pm$ 0.45	0.12 $\pm$ 0.00	+ 50.6 $\pm$ 3.5	-----
SUV <sub>cat</sub> VAN	188.8 $\pm$ 3.92*	0.25 $\pm$ 0.01*	+72.5 $\pm$ 5.6 *	10.08 $\pm$ 0.14

T teste, \* $P<0.05$ .

The release kinetics of plain and VAN-containing liposomal formulations was determined *in vitro*. As seen in **Figure 2**, encapsulated VAN formulations showed more prolonged release overtime as compared to VAN-free formulations ( $P<0.05$ ).

The SUV VAN group showed a more sustained, slower release profile than the other liposomes ( $P<0.05$ ), whereas SUV<sub>fuso</sub> VAN and SUV<sub>cat</sub> VAN were found to have a very similar release kinetics compared to each other ( $P>0.05$ ). As expected, VAN-free formulations showed greater percent release at all timepoints, with a significant difference from the other liposomal formulations ( $P<0.05$ ).



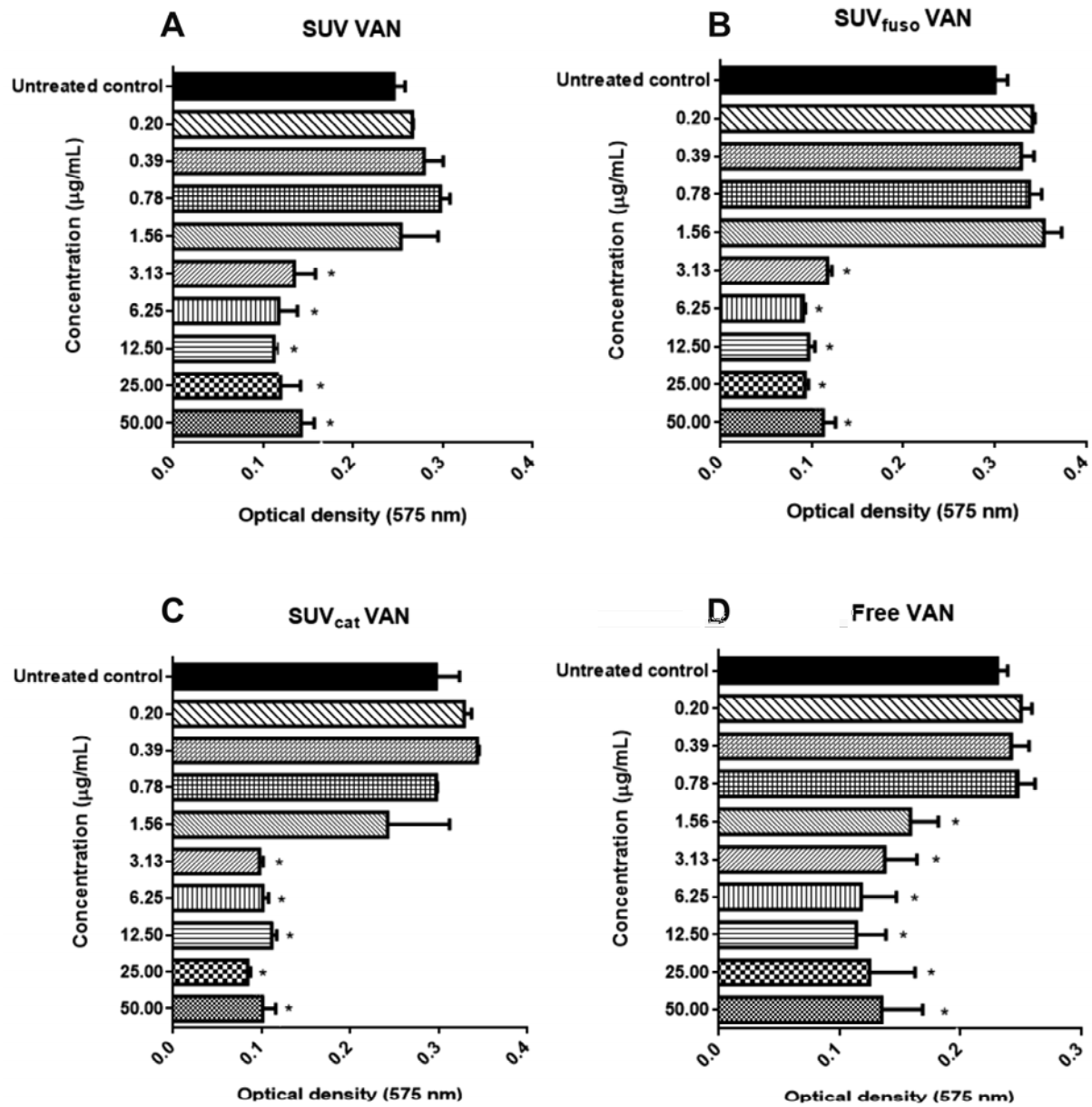


**Figura 2.** Mean ( $\pm$  SD) of the percent release of free and liposome-encapsulated VAN for 12h. Two-way ANOVA,  $*P < 0.05$ . The letters indicate statistical difference between groups, as follows: a) SUV VAN x SUV<sub>fuso</sub> VAN – 1 to 12 h; b) SUV VAN x SUV<sub>cat</sub> VAN– 1 to 12h; c) SUV VAN x free VAN – 0.15 to 12 h; d) SUV<sub>fuso</sub> VAN x free VAN – 0.15 to 8h; e) SUV<sub>cat</sub> VAN x free VAN – 0.15 to 8h.

### Antimicrobial Activity

Free and liposome-encapsulated VAN formulations affected bacterial growth in both MSSA and MRSA strains, with MIC values ranging between 0.78 and 1.56 µg/mL. These findings are in line with the information provided by the CLSI concerning *S. aureus* susceptibility to VAN (CLSI, 2012).

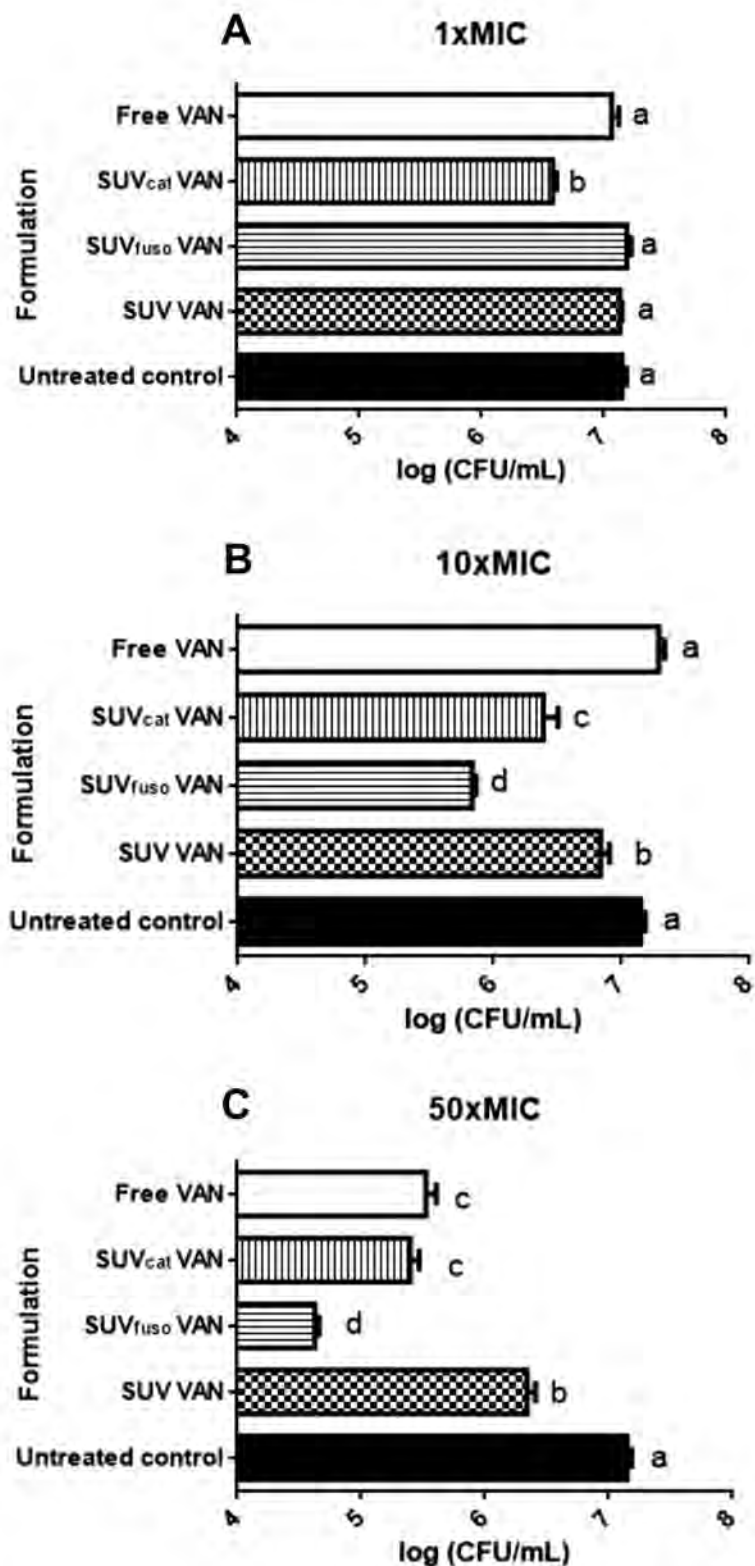
The formulations were next tested for their inhibitory effects on *S. aureus* ATCC 29213 biofilm adherence and formation. As shown in **Figure 3**, treatment with all formulations inhibited biofilm formation in a dose-dependent fashion. Free VAN was found to inhibit biofilm formation from MIC (1.56 µg/mL) and higher concentrations as compared to the untreated biofilm control, while the inhibitory effects of liposome-encapsulated VAN were only seen from 2xMIC (3.13 µg/mL). These results corroborate those of the *in vitro* release kinetics assay (**Figure 2**), in which encapsulated VAN showed a late release profile as compared to free VAN. Thus, it is likely that a lower amount of VAN molecules was initially released from the liposomal formulations, thereby slowing up their overall antimicrobial effects.



**Figure 3.** Mean ( $\pm$  SD) optical density values of *S. aureus* biofilms treated with different concentrations of VAN encapsulated into SUV VAN (A), SUV<sub>fuso</sub> VAN (B), SUV<sub>cat</sub> VAN (C), or free VAN solution (D). The asterisk “\*” indicates statistically significant difference between the drug treatment and its respective untreated control at  $P < 0.05$  (One-way ANOVA, followed by Tukey’s post-hoc test).

The inhibitory effects of the formulations on biofilm viability were also investigated. **Figure 4** shows the mean ( $\pm$  SD) CFU/mL ( $\text{Log}_{10}$ ) of biofilms treated for 24 h at 1xMIC, 10xMIC, and 50xMIC. The data were compared between treatment groups and the untreated control. At 1xMIC, only SUV<sub>cat</sub> VAN caused a significant decrease in the number of viable biofilm cells ( $P < 0.01$ ). Nevertheless, at 10xMIC and 50xMIC all formulations showed significant inhibitory effects as compared to the untreated control ( $P < 0.05$ ). Free VAN was not able to affect biofilm viability significantly at 10xMIC ( $P > 0.05$ ), but did at 50xMIC ( $P < 0.05$ ). When liposomal formulations were compared among themselves, we observed that SUV<sub>fuso</sub> VAN had the most accentuated inhibitory potential on mature biofilms, followed by SUV<sub>cat</sub> VAN and SUV VAN, with significant differences between them ( $P < 0.05$ ).

The effects on mature biofilms treated with SUV<sub>cat</sub> VAN and free VAN were found to be similar at 50xMIC ( $P > 0.05$ ) and greater than those promoted by SUV VAN ( $P < 0.05$ ). SUV<sub>fuso</sub> VAN was the most active formulation against *S. aureus* biofilm viability when compared to the other groups ( $P < 0.05$ ). SUV<sub>fuso</sub> VAN reduced biofilm viability by 3.5  $\text{Log}_{10}$  CFU/mL (35x); SUV<sub>cat</sub> VAN and free VAN caused a reduction of 2.5  $\text{Log}_{10}$  CFU/mL (25x), while SUV VAN reduced biofilm viability by 1  $\text{Log}_{10}$  CFU/mL (10x) as compared to the control.



**Figure 4.** Inhibitory effects of liposomal and plain formulations on *S. aureus* ATCC 29213 mature biofilm viability at 1xMIC (A), 10xMIC (B), and 50xMIC (C). The values are expressed as mean ( $\pm$  SD) of CFU/mL. The asterisk “\*” indicates a statistically significant difference at  $P < 0.05$  (One-way ANOVA, with Tukey’s post-hoc test).

## Discussion

Nosocomial and community-acquired MRSA infections remain a major concern in global health and have driven the adoption of public policies and medical research in this field (Holland et al., 2014; Honary et al., 2013; Elkodairy et al., 2014). Evidence has shown the promising results of liposomal vesicles as drug carriers for pharmaceutical application (Kim et al., 1999; Kadry et al., 2004; Nicolosi et al., 2010; Ma et al., 2011; Moghadas-Sharif et al., 2015; Nicolosi et al., 2015). Herein, we report the development, characterization, and antimicrobial properties of experimental formulations containing VAN encapsulated into conventional, fusogenic and cationic liposomes. We demonstrated that the experimental formulations were more active than VAN-free ones in reducing mature biofilm, with better efficacy for SUV<sub>fuso</sub> VAN.

Our goal when selecting the liposomal formulations was to achieve greater interaction with bacterial cells and, thereby, facilitate penetration into mature biofilms. SUV liposomes contained a mixture of EPC, a zwitterionic lipid that facilitates electrostatic bonds with the drug, in addition to cholesterol, which increases the rigidity and stability of the vesicles (de Paula et al., 2012). SUV<sub>fuso</sub> liposomes contained the lipid DOPE in their composition, which (i) promotes destabilization of the liposomal vesicle at acidic pH (due to its inverse hexagonal structure), (ii) increases lipophilicity, and (iii) reduces interaction energy in the lipid bilayer. The use of DPPC was required for formation and stabilization of the lipid bilayers due to DOPE instability. The lipids EPC and CHEMS contribute to greater stability of the formulation (Nicolosi et al., 2010; Aoki et al., 2015; Nicolosi et al., 2015). SUV<sub>cat</sub> liposomes contained Sa, EPC, and cholesterol in their composition. Sa is a positively charged lipid that facilitates adsorption through electrostatic interactions with the negatively charged biofilms (Balazs and Godbe, 2011). In order to prevent lipid oxidation, the antioxidant alpha-tocopherol was added to all liposomal formulations (de Paula et al., 2012).

Encapsulation of VAN altered the size, modular electrical charge of the vesicles, and system homogeneity in all liposomal formulations, as there was an increase in size and PDI values when comparing plain and VAN-containing liposomes (Table 1). Such increase in vesicle size and PDI may be due to the high molecular weight and hydrophilicity of the VAN molecule (Liu et al., 2015; Srinivas et al., 2015). Hence, it is possible that encapsulated VAN has a tendency to be located

in the aqueous core or adjacent lipid-water interface near the polar head groups (Bozzuto and Molinari, 2015). This results in increased vesicle size and reduction of its homogeneity upon drug encapsulation. Similar results concerning vesicle size and PDI were found in another study with conventional liposomes containing EPC and cholesterol (7:1) and cationic liposomes (EPC:Sa:Chol, 7:2:1), both prepared with 20 mg/mL VAN (Kadry et al., 2004). This similarity may be explained by the use of the same method for liposome preparation used in our study. Another study with fusogenic liposomes showed similar findings with regard to vesicle size, but lower PDI value (0.037) (Nicolosi et al., 2010). The liposome composition was very similar to that of our study, and the formulation was prepared by the reverse-phase evaporation method with manual extrusion in 100 nm-pore membranes containing 9 mg/mL VAN. Some authors have suggested that the homogeneity of liposomal systems depends on the preparation method and drug concentration used in the formulations (Moghadas-Sharif et al., 2015).

Encapsulation of VAN decreased the zeta potential in SUV VAN and SUV<sub>fuso</sub> VAN, which suggests lower stability of these formulations. On the other hand, SUV<sub>cat</sub> VAN presented higher zeta potential, which might indicate greater stability of the vesicles after encapsulation with VAN as compared to the other liposomes (Mohanraj and Chen, 2006). The zeta potential values found in our study in SUV<sub>cat</sub> VAN are in accordance with the findings reported in the literature on cationic liposomes composed of a lipid mixture of DPPC:Sa:Chol (1:0.49:0.81 mol%), prepared by the same method and containing the same VAN concentration of that in the present study (Kim et al., 1999).

Morphological analysis of liposomes revealed the presence of spherical vesicles, with a single lipid bilayer (Figure 1) around 200 nm in diameter, which is in agreement with the dynamic light scattering data. Some vesicles in the SUV<sub>fuso</sub> formulations were found to be in close contact with each other, which represents the ability of liposomes to merge (Nicolosi et al., 2010; Aoki et al., 2015; Nicolosi et al., 2015).

In our study, the liposomes SUV VAN and SUV<sub>cat</sub> VAN showed greater %EE (32.46% and 10.08%, respectively) than those reported elsewhere in the literature (2% and 5%, respectively) using the same types of liposomes prepared by sonication with similar composition and VAN at 20 mg/mL (Kadry et al., 2004). Previously,

Nicolosi et al. (2010) observed greater %EE (65.8%) of fusogenic liposomes – prepared by the reverse-phase evaporation method – as compared to our findings (11.04%). According to the authors, the preparation method and drug concentration in the liposomal suspension may have influenced the different %EE results obtained (Muppidi et al., 2012; Liu et al., 2015).

In this study, no significant difference was observed in the release kinetics of VAN-containing SUV<sub>fuso</sub> and SUV<sub>cat</sub>. Both formulations released 12% of VAN after 1 h, whereas SUV VAN released 2% and free VAN, 33%. The differences in the drug controlled release profile among the liposomal formulations may be a result of their diverse %EE (Liu et al., 2015). Recently, Srinivas et al. (2015) evaluated the release kinetics of VAN from conventional liposomes containing EPC (50 mg), Chol (20 mg) and VAN (10 mg/mL), prepared by the ethanol injection method. The authors observed similar results to those found in our study with regard to VAN release from SUV VAN liposomes, and different results with regard to release of free VAN, which was about 42% after 22 h. This divergence may be related to the free VAN concentration used in the donator compartment, which was 100 mg/mL in the study by Srinivas et al. (2015) and 10 mg/mL in our study. Another *in vitro* study reported different results from those presented herein with regard to the release kinetics of VAN from conventional liposomes or free VAN solution (Liu et al., 2015). The authors found release percentage of 31% after 1 h and 75% after 4 h, respectively. In this previous study, the conventional liposomes were composed of Chol:EPC (1:4 mol%) containing VAN:lipids (1:15 mol%) prepared by the reverse-phase evaporation method. The differences in the release profile may be explained by their composition, preparation method and %EE (40.31%) of the formulations.

We encapsulated VAN into different types of liposomes in order to carry out a comparative analysis of its efficacy on *S. aureus* biofilms in relation to free VAN. VAN exerts antibacterial action by inhibiting the synthesis of cell wall peptidoglycans (Howden et al., 2010; Ng et al., 2011; Elkhodairy et al., 2014). This drug has a high affinity to the residue D-Ala-D-Ala from the peptidoglycan precursor, lipid II, thereby blocking the addition of final precursors by transglycosylation and transpeptidation, which ultimately interrupts cell wall formation. In *S. aureus*, peptidoglycan biosynthesis takes place in the cell division septum in a specific site of the cytoplasmic membrane (Howden et al., 2010). Thus, in order to promote its effects on



the cell wall, VAN molecules should penetrate approximately 20 layers of peptidoglycan to reach the division septum and bind to the protein fraction (L-lysine-D-alanyl-D-alanine) of murein monomers used as substrate for glycosyltransferases. Depending on the bacterial cell cycle phase, the division septum can be completely formed or under formation (Nicolosi et al., 2010). Hence, the distance between the cell wall and the plasma membrane is shorter at the beginning of bacterial growth, which might have contributed to the bactericidal effects of free VAN. However, when bacterial growth reaches a final stage, the division septum is completely formed. As a result, the distance between the cell wall and the plasma membrane is wider, which may hinder the action of free VAN. In this case, it is believed that encapsulated VAN could more effectively penetrate the cell wall and reach the periplasmic space, therefore promoting its antibacterial effects (Howden et al., 2010; Nicolosi et al., 2010; Sande et al., 2012). Such increased penetration can explain the better antibiofilm activity observed in our study for liposomal formulations.

The MIC values of SUV VAN, SUV<sub>fuso</sub> VAN and SUV<sub>cat</sub> VAN and free VAN and their inhibitory effects on biofilm formation were found to be similar, as bacterial strains were used at early stages of biofilm development. On mature biofilms, however, encapsulated VAN showed greater bactericidal effects due to its increased ability to penetrate the peptidoglycan layers, whereas free VAN remained trapped in the cell wall. The MIC values of liposome-encapsulated VAN on *S. aureus* ATCC 29213 observed in our study are in agreement with those found by Kadry et al. (2004). These authors reported MIC values of 0.75 µg/mL and 1.50 µg/mL for cationic and conventional liposomes, respectively. Another study found that encapsulation of VAN into conventional liposomes reduced by 2 the drug MIC against MRSA strains as compared to free VAN (Sande et al., 2012). This liposome formulation was composed of DSPC:DCP:Chol (7:2:1, mol%) containing VAN at 50 mg/mL, which was 5 times higher than the VAN concentration used in our study.

Our findings indicate that free VAN at MIC had better inhibitory effects on early stages of biofilm formation than had liposome formulations. The latter inhibited biofilm adherence only from 2xMIC, probably due to the slower drug release from the liposomes. During the first thirty minutes, free VAN solution released 21% of its drug content while the liposomes SUV VAN, SUV<sub>fuso</sub> VAN and SUV<sub>cat</sub> VAN released about 1%, 5% and 3%, respectively. After 4 h, SUV<sub>cat</sub> VAN and SUV<sub>fuso</sub> VAN released 47%

and 40% of their drug content, respectively, comparable to that of free VAN (54%). Thus, we believe that the lower amount of VAN released at the first treatment hours significantly influenced biofilm formation. For instance, the lower amount of VAN available from the liposomes may have allowed more robust biofilm formation in relation to free VAN-treated groups. On the other hand, the liposomal formulations showed better antibacterial activity than free VAN against mature biofilms, being SUV<sub>fuso</sub> VAN the most active one, followed by SUV<sub>cat</sub> VAN. Cationic liposomes may have a higher affinity for negatively charged biofilms, which can decrease VAN delivery time into the infectious focus (Kadry et al., 2004; Kim et al., 1999). Accordingly, these liposomes probably release VAN in the proximities of the bacterial cell wall due to the affinity with its negative charge, resulting in inhibition of cell wall biosynthesis. Fusogenic liposomes have an increased potential to interact with extracellular matrix and cell wall due to their ability to merge with lipid membranes (Nicolosi et al., 2010). These vesicles can pass through the cell wall and deliver VAN into the periplasmic space, thereby making it easier for the drug to reach the division septum and block peptidoglycan biosynthesis (Howden et al., 2010; Nicolosi et al., 2010; Sande et al., 2012).

This is the first study evaluating the antibacterial power of these formulations on biofilm cultures. Other reports in the literature have also confirmed that liposome-encapsulated VAN significantly affects bacterial cell viability as compared to free VAN. An *in vivo* study (Kadry et al., 2004) with rabbits tested conventional, cationic and anionic VAN-containing liposomes for the treatment of chronic osteomyelitis caused by *S. aureus*. The authors observed therapeutic efficacy for encapsulated VAN formulations, in contrast with what was observed for the free VAN group, which did not present significant effects. In addition to the advantageous electrostatic attraction of cationic liposomes, these remain in the bloodstream for a longer time – since they are slowly absorbed by macrophages in the liver and spleen – and therefore promote bactericidal effects with lower doses of the drug (Kadry et al., 2004). An *in vitro* study investigated the adsorption capacity and antimicrobial activity of VAN-containing and plain cationic liposomes on *S. aureus* biofilms and compared to the effects of free VAN. The authors found that cationic vesicles can have a high adsorption capacity onto bacterial cells and exert antimicrobial activity on preformed biofilms (Kim et al., 1999). In another *in vitro* study, the authors developed an artificial

bone model impregnated with VAN-containing or plain cationic liposomes for the treatment of *S. aureus* infections. They observed better antibiofilm activity of cationic liposome-encapsulated VAN when compared to free VAN (Ma et al., 2011). Collectively, the results of these previous reports corroborate those found in our study.

Herein, we showed that experimental SUV<sub>fuso</sub> VAN and SUV<sub>cat</sub> VAN formulations had satisfactory features showed antimicrobial activity greater than that of unencapsulated VAN and SUV VAN liposomes. Further research should evaluate the antimicrobial activity of these formulations against other bacterial strains as well as their therapeutic efficacy in animal models and clinical trials.

## Conclusion

Cationic and fusogenic liposomal formulations containing vancomycin were successfully developed and characterized in this study. In all formulations, encapsulated vancomycin showed better antibacterial efficacy *in vitro* as compared to free vancomycin against *S. aureus* preformed biofilms. Thus, cationic and fusogenic liposomes containing vancomycin can be considered promising antibacterial agents for *S. aureus* infection control.

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### 3 CONCLUSÃO

As formulações de lipossomas catiônicos e fusogênicos contendo vancomicina foram desenvolvidas e caracterizadas com sucesso. A vancomicina encapsulada em todas as formulações lipossomais apresentou maior eficácia antibacteriana *in vitro* em relação à vancomicina livre em biofilme formado por *S. aureus*. Portanto, lipossomas catiônicos e fusogênicos contendo vancomicina podem ser considerados promissores agentes antibacterianos na terapêutica de infecções causadas por *S. aureus*.

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## ANEXO 1 – Confirmação de submissão do artigo

AAC01843-16: Manuscript Received





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Adicionar aos contatos 24/08/2016

Ações ▾

Para: abscriboni@hotmail.com ✉

Dear Ms. Borges Scriboni,

On August 24, 2016, we received the manuscript "**Characterization and antibiofilm activity of liposome-encapsulated vancomycin against *Staphylococcus aureus*.**" by Andreia Borges Scriboni, Veronica Muniz Couto, Irlan Almeida Freires, Francisco Groppo, Eneida de Paula, Michelle Franz Montan Braga Leite, Karina Cogo Müller, and Ligia Nunes de Moraes Ribeiro. The submission form indicates that this paper should be processed as a(n) Full-Length Text intended for publication in the section Pharmacology.

The manuscript has been assigned the control number AAC01843-16. Take note of this number, and refer to it in any correspondence with the Journals Department or with the editor.

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All authors must disclose any commercial affiliations as well as consultancies, stock or equity interests, and patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript. All funding sources for the project, institutional and corporate, and any potentially conflicting interests, such as relationships that might detract from an author's objectivity in presentation of study results, must be acknowledged, both in the Acknowledgments section and on this form. The corresponding author must review this policy with all coauthors.

The author submitting the manuscript must state in the submission form whether or not any of the authors has a conflict of interest. Here is how Ms. Borges Scriboni responded: